Running head: Pyoverdine impact on plant growth/defense balance

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The *Pseudomonas fluorescens* siderophore pyoverdine weakens *Arabidopsis thaliana* defense in favour of growth in iron-deficient conditions

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Summary of the most important findings:
Pyoverdine, a high-affinity ferric iron chelator synthesized by *Pseudomonas fluorescens*, impacts the growth/defense trade-off of *Arabidopsis thaliana* in iron deficiency conditions

FOOTNOTES

Author contributions
P.T., L.A., S.M., P.L., D.W. and A.B.B. conceived the original project; P.T. performed most of the experiments with the help of L.A. and A.B.B.; A.K. provided technical assistance; P.T., L.A., D.W. and A.B.B. designed the experiments and analyzed the data; S.P. conducted the CATMA microarray analysis; S.C. analyzed the samples for hormone contents except ethylene; C.C. analyzed the samples for ethylene contents; A.B.B. wrote the article with the contribution of DW.
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ABSTRACT

Pyoverdines are siderophores synthesized by fluorescent pseudomonas spp. Under iron-limiting conditions, these high-affinity ferric iron chelators are excreted by bacteria in the soil to acquire iron. Pyoverdines produced by beneficial Pseudomonas ameliorate plant growth. Here, we investigate the physiological incidence and mode of action of pyoverdine from P. fluorescens C7R12 on Arabidopsis thaliana plants grown under iron-sufficient or -deficient conditions. Pyoverdine was provided to the medium in its iron free structure (apo-pyoverdine), thus mimicking a situation in which it is produced by bacteria. Remarkably, apo-pyoverdine abolished the iron deficiency phenotype and restored the growth of plants maintained in the iron-deprived medium. In contrast to a P. fluorescens C7R12 strain impaired in apo-pyoverdine production, the wild-type C7R12 reduced the accumulation of anthocyanins in plants grown in iron-deficient conditions. Under this condition, apo-pyoverdine modulated the expression of around 2000 genes. Notably, apo-pyoverdine positively regulated the expression of genes related to development and iron acquisition/redistribution while it repressed the expression of defense-related genes. Accordingly, the growth promoting effect of apo-pyoverdine in plants grown under iron-deficient conditions was impaired in iron-regulated transporter 1 (irt1) and ferric chelate reductase 2 (fro2) knock-out mutants and was prioritized over immunity as highlighted by an increased susceptibility to Botrytis cinerea. This process was accompanied by an over-expression of the transcription factor HBI1, a key node for the cross-talk between growth and immunity. This study reveals an unprecedented mode of action of pyoverdine in Arabidopsis and demonstrates that its incidence on physiological traits depends on the plant iron status.
Iron, the fourth most abundant element in the Earth crust, is an essential micro-nutrient for plant growth and development, notably through its involvement in major metabolic processes such as respiration and photosynthesis (Guerinot & Yi, 1994; Robin et al., 2008). Despite its abundance, iron is weakly bioavailable for organisms due to its poor solubility in soils under aerobic conditions. Therefore, plants and other organisms had to evolve mechanisms to efficiently assimilate iron from the soil. Bacteria, but also other microbes including fungi, produce iron chelators known as siderophores (Ahmed and Holmström, 2014). Siderophores are low molecular weight molecules that exhibit a high affinity for ferric iron ($Fe^{3+}$) and are synthesized in iron-limiting conditions. For instance, fluorescent *pseudomonas* spp. produce yellow-green fluorescent siderophores termed pyoverdines (or pseudobactins) having a high affinity for $Fe^{3+}$ with a stability constant of ferric-pyoverdine complex showing around $10^{32}$ M$^{-1}$ (Meyer & Abdallah, 1978; Albrecht-Gary et al., 1994). Once excreted, siderophores chelate ferric iron and are transported back as ferric-siderophore complexes inside the bacterial cell where ferric iron is released from the siderophore and further reduced to ferrous iron ($Fe^{2+}$) (Chu et al., 2010).

Higher plants evolved two main strategies to incorporate iron (Curie and Briat, 2003). Grasses plants use a strategy similar to the bacterial one, based on the release of phytosiderophores (PS, strategy II). Non grass plants, like *Arabidopsis thaliana*, use the strategy I based on soil acidification through proton excretion by H$^+$-ATPases, solubilisation of $Fe^{3+}$ and reduction of the $Fe^{3+}$-chelate mainly by the ferric chelate reductase FRO2. $Fe^{2+}$ ions, which are more soluble than $Fe^{3+}$, are subsequently taken up by root transporters including IRT1 (Iron-Regulated Transporter 1). Once in the plants, iron has to be transported, utilized or sequestrated because of its high reactivity and toxicity through the Fenton reaction. *IRT1* and *FRO2* expression is induced under iron limiting conditions and the transcription factor FIT1 is required for a proper regulation of the iron uptake system in *A. thaliana* (Colangelo & Guerinot, 2004). FIT1 does not act alone to orchestrate the iron deficiency response. Indeed, several studies highlighted the role of other bHLH transcription factors on the expression of genes involved in the adaption to iron deficiency stress. These transcription factors were shown to act together with FIT1 such as bHLH38 and bHLH39 (Yuan et al., 2008), or alone as reported for POPEYE (PYE), bHLH100 and bHLH101 (Long et al., 2010; Sivitz et al., 2012). Besides the FRO2/IRT1 system, Fe-efficient plants also respond to Fe deficiency by
enhancing the root secretion of coumarin compounds improving plant iron nutrition (Fourcroy et al., 2014).

Because iron is central for many metabolic processes and not easily available, it is the focus of serious competition between organisms. In mammals, there are several examples in which the virulence of infectious bacteria has been correlated to their ability to assimilate host iron through their siderophores (Saha et al., 2013). In turn, the host fights back and develops strategies to avoid iron capture by bacteria, highlighting cross-regulatory interactions between iron homeostasis and immune responses (Ong et al., 2006; Nairz et al., 2014). Similar processes seem to operate in plants. For example, the production of siderophores by the pathogenic enterobacteria *Dickeya dadantii*, *Erwinia amylovora* and *Pseudomonas syringae* pv. *tabaci* was shown to facilitate infection in their hosts, pointing out their role as virulence factors (reviewed in Franza & Expert, 2013). Consecutively, plants establish a strategy for keeping iron which consists in lowering iron availability by overexpressing iron-chelating proteins, notably ferritins, and in improving iron acquisition through IRT1 (Dellagi et al., 2005 and 2009). As highlighted in animals, this iron retention strategy used by plants to combat infections could be a key component of their immune responses. Supporting this concept, treatment of plant tissues or cell suspensions by bacterial siderophores was reported to trigger defense responses in tobacco and *A. thaliana* (van Loon et al., 2008; Aznar et al., 2014).

On the other hand, siderophores secretion by bacteria is not always associated to pathogenicity and, in contrary, is even beneficial for plant health. In particular, different soil pseudomonad strains, commonly called Plant Growth-Promoting Rhizobacteria (PGPR), were shown to suppress soilborne diseases (Haas et al., 2005; Weller et al., 2007). Their biocontrol activities have been correlated to their capacity to release siderophores which act as competitors reducing the iron availability for plant pathogens including for instance *Fusarium oxysporum* and *Pythium ultimum* (Lemanceau et al., 1992, 1993; Duijff et al., 1999; Bakker et al., 2007; Van Wees et al., 2008; Ahmed and Holmström, 2014). Importantly, siderophores produced by other bacterial species such as *Bacillus subtilis* also display biocontrol activities (See for instance Yu et al., 2011). Beside their ability to compete with soil pathogens for iron, siderophores produced by plant beneficial rhizobacteria were also shown to protect different plant species by eliciting induced systemic resistance (ISR). For example, purified pyoverdines from *Pseudomonas putida* WCS358 were shown to trigger ISR in *A. thaliana* when using *P. syringae* pv. *tomato* as the challenging pathogen. They were also efficient in inducing ISR against *B. cinerea* in bean and in tomato (Meziane et al., 2005). Accordingly,
De Vleesschauwer et al. (2008) demonstrated that pyoverdine was the determinant responsible for *P. fluorescens* WCS374-induced ISR in rice against the leaf blast pathogen *Magnaporthe oryzae*. Investigations on tobacco cell suspensions indicated that the pyoverdine siderophores from *P. putida* WCS358 and *P. fluorescens* WCS417 strains are perceived by cells and mediate defense-related early signaling events (Van Loon et al., 2008). However, these defense-related reactions were poorly correlated to the ability of the siderophores of interest to promote ISR. Therefore, how siderophores promote ISR remains poorly understood.

In addition to their protective function, siderophores produced by PGPR were shown to enhance plant growth, probably by providing the plants with nutrients. Of particular interest here, Vansuyt et al. (2007) provided evidence that in its iron-chelated structure, pyoverdine from *P. fluorescens* strain C7R12 is assimilated by roots of *A. thaliana* plantlets grown *in vitro*. This process was accompanied by an improvement of plant growth and chlorophyll content. This study also revealed that iron chelated to pyoverdine was incorporated in a more efficient way than chelated to EDTA. The mechanism by which siderophores enhance iron nutrition is little known. It has been proposed that microbial ferric-siderophore could be reduced to release ferrous iron once transported in the apoplast of root cells, thus providing substrate for the iron transport system (Ahmed and Holmström, 2014). Also, ferric-siderophore could provide iron to plant phytosiderophores (Crowley, 2006). In the case of pyoverdine, Vansuyt et al. (2007) provided another explanation by showing that, in *A. thaliana*, pyoverdine is incorporated by plant roots and accumulates in shoots. Interestingly, this incorporation do not rely on the strategy I iron acquisition system. Taken together, these observations underline the importance of pyoverdine in plant-microbe interactions but call for further studies to better understand the mechanisms involved.

In the present study, we further deciphered the mode of action of pyoverdine in plants. For this purpose, we investigated the incidences of pyoverdine from *P. fluorescens* C7R12 on the growth and defense capacities of *A. thaliana* plantlets grown in iron-sufficient or in iron-deficient medium. Pyoverdine was provided to the culture medium in its iron free structure (apo-pyo), thus mimicking a situation in which it is produced by the bacterial strain. We observed that while apo-pyo displayed a remarkable growth promoting effects on plants facing iron deficiency, it clearly impaired their resistance against *Botrytis cinerea*. Contrary to a *P. fluorescens* C7R12 bacterial strain impaired in apo-pyo production, the wild-type C7R12 reduced the accumulation of anthocyanins in plants grown under iron-deficient conditions. Analysis of the underlying mechanisms showed that the apo-siderophore triggered a strong
up-regulation of genes related to development and iron uptake/redistribution in planta. Accordingly, the growth promoting effects of apo-pyo was shown to rely on \textit{IRT1} and \textit{FRO2} expression. Furthermore, we provide evidence that in plants exposed to iron deficiency, the apo-siderophore modulates the expression of genes controlling the trade-off between growth and immunity in favour of growth. Collectively, data from our work describe an unexpected mode of action of pyoverdine in \textit{A. thaliana} plants grown in iron deficiency.
RESULTS

1. Impact of apo-pyoverdine on *A. thaliana* phenotype

Four week-old plants were subjected to apo-pyo treatment in iron-deficient or iron-sufficient medium as described in Fig. 1. For this purpose, after 4 weeks of hydroponic culture in a classical nutrient solution containing 25 µM Fe-EDTA, plants were subjected to 24 h pre-treatment in iron-sufficient (nutritive solution with 25 µM Fe-EDTA) or iron-deficient medium (nutritive solution without Fe-EDTA). Then, each resulting batch was separated in two new batches containing fresh nutritive solution with or without 25 µM apo-pyo so as to obtain four different treatments: 25 µM apo-pyo in iron-sufficient (Fe 25 apo-pyo) or in iron-deficient medium (Fe 0 apo-pyo) and the corresponding controls without apo-pyo (referenced Fe 25 or Fe 0 according to the presence or absence or Fe-EDTA, respectively). Importantly, we measured the iron content of the culture media 6 h after apo-pyo addition (see below, Fig 6A). The corresponding data showed that apo-pyo addition did not result in iron enrichment in the medium, thus proving that the siderophore was purified in its iron-free structure and was not contaminated by ferri-pyo.

First, we checked that in our experimental conditions apo-pyo was efficiently assimilated by plants and potentially transported from roots to shoots. The presence of pyoverdine in root tissue was investigated by ELISA using rabbit polyclonal anti-pyoverdine antibodies. In root extracts of Fe 25 apo-pyo- and Fe 0 apo-pyo-treated plants, we detected 0.64 and 0.82 ng of pyoverdine per µg of proteins, respectively (Fig. 2A). In shoots, we were not able to discriminate a positive signal from the background (not shown). To strengthen the data, the presence of apo-pyo in plant tissues was further analyzed by isotope ratio mass spectrometry. Following the treatment of plants by 15N-apo-pyo, approximately 4.5 µmol of 15N per hour per g of dry weight (DW) was measured in the roots of Fe 25 apo-pyo- and Fe 0 apo-pyo-treated plants, thus confirming the results from the ELISA assays. In addition, a lower signal (0.37 and 0.54 µmol of 15N/h/g of DW for Fe 25 apo-pyo- and Fe 0 apo-pyo treatments respectively) was also measured in the shoots (Fig. 2B). These data also showed that the concentration of apo-pyo assimilated by plants did not differ according to the concentration of iron in the culture medium.

Next, the impact of apo-pyo treatment on the plants macroscopic phenotype was evaluated (Fig. 3). Fe 25- and Fe 25 apo-pyo-treated plants appeared bigger and greener as compared to plants facing iron deficiency. As expected, plants facing Fe 0 treatment were severely affected after 7 days of culture: they showed a reduced growth and their leaf abaxial sides displayed a
violet pigmentation probably due to anthocyanin production (Fig. 3A). In comparison to Fe 0-
treated plants, Fe 0 apo-pyo-treated plants exhibited a distinct phenotype which resembled
those of plants grown in iron-containing medium. Indeed, after 7 days of treatment with apo-
pyo, their growth was considerably higher. Quantitatively, their root masses were in between
those of Fe 25/Fe 25 apo-pyo and Fe 0 treated plants (Fig. 3B) and growth of their aerial parts
was similar to those of Fe 25 plants (Fig. 3C). Furthermore, although we noticed a yellowing
of several leaves, Fe 0 apo-pyo-treated plants did not show violet pigmentation as compared
to Fe 0-treated plants (Fig. 3A). Supporting this observation, the high production of
anthocyanins measured in Fe 0 plants was not observed in Fe 0 apo-pyo-treated plants which,
in contrast, displayed an anthocyanin level similar to those of Fe 25- and Fe 25 apo-pyo-
treated plants (Fig. 3D).

Collectively, these data indicate that apo-pyo is assimilated by *A. thaliana* plants grown in
hydroponic conditions and highlight a growth-promoting effect of the apo-siderophore in
plants facing iron deficiency.

2. Transcriptomic analysis of apo-pyo-treated plants

We initiated a transcriptomic analysis in order to provide a first and overall view of how apo-
pyo impacts the growth of plants. For this purpose, a microarray analysis using CATMA
arrays covering the entire transcriptome of *A. thaliana* was performed from roots and shoots
of plants 3 days after the addition of apo-pyo in the nutritive medium containing (Fe 25 apo-
pyo condition) or not (Fe 0 pyo condition) iron. This time point was chosen on the basis of
Fig. 3B and 3C demonstrating that the increase of growth of the Fe 0 apo-pyo plants was
noticeable from 3 days. Changes in root and shoot gene expression were determined by
comparing the transcriptomes of apo-pyo-treated plants with their respective controls non-
treated with the apo-siderophore (that is Fe 25 apo-pyo *versus* Fe 25; Fe 0 apo-pyo *versus* Fe
0).

We first selected genes whose expression was modulated by a log 2 ratio ≥ + 1.5 or ≤ - 1.5
(with adjusted p-value ≤ 0.05). This analysis revealed that 2109 genes displayed significant
differential expression in response to apo-pyo (Fig. 4A, listed in Tables S1 to S4). The
incidence of apo-pyo on gene expression clearly differed according to the tissue and the
presence or not of iron in the culture medium. Indeed, apo-pyo modulated the expression of
2053 genes in iron-depleted medium (Fig. 4B) *versus* 136 genes in iron-containing medium
(Fig. 4C). Furthermore, 1837 genes showed a modified expression in the roots (Fig. 4D)
*versus* 361 in the shoots (Fig. 4E). Regarding the percentages of induced/repressed genes,
apo-pyo-induced genes represent 55 to 56% of the total apo-pyo-modulated genes when considering the roots (Fig. 4D), the shoots (Fig. 4E) or the Fe 0 condition (Fig. 4B). In contrast, in plants grown in the Fe 25 medium, 90% of the genes were induced in response to the apo-pyo treatment (Fig. 4C).

A closer analysis indicate that 79 of the apo-pyo-dependent genes (genes numbered 1 to 79, Table S5) could be gathered in a first cluster corresponding to genes modulated under iron deficiency in both roots and shoots. Amongst these genes, 22 (28%) and 39 (49%) were induced or repressed in both tissues, respectively (Fig. 4B). A second cluster includes 63 genes (genes numbered 2, 62, 75 and 80 to 138, Table S5) modulated in roots in iron-deficient and iron-sufficient media. Amongst this category, 45 (71%) were inversely regulated according to the presence or absence of iron in the culture medium. The third cluster groups 10 shoot genes (genes numbered 49, 60, 61, 71 and 139 to 144, Table S5) which showed differential expression in iron-sufficient and iron-deficient medium, 9 of them being inversely modulated in the two conditions. Therefore, a large part of the genes modulated by apo-pyo in roots and shoots of plants cultivated in iron-deficient condition were similarly regulated. On the contrary, a majority of the apo-pyo-dependent genes modulated in both iron-sufficient and in iron-deficient conditions were regulated in the opposite way. Using AmiGO (http://amigo1.geneontology.org/cgi-bin/amigo/term_enrichment), we identified these genes as mainly responsive to stress or to stimulus.

Next, we focused our analysis on the genes whose expression was highly induced (91 and 15 genes in roots and shoots, respectively) or highly repressed (94 and 8 genes in roots and shoots, respectively) by apo-pyo in the iron-deficient condition (log 2 ratio ≥ +3 or ≤ -3; p-value ≤ 0.05). This choice was made based on the phenotypic analysis showing that apo-pyo deeply improved the growth of plants facing iron deficiency (Fig. 3). Genes were putatively assigned to functional categories based on the Gene Ontology Annotations from the TAIR database (http://www.arabidopsis.org/tools/bulk/go/index.jsp; Fig 5). Concerning the genes whose expression was induced by apo-pyo, an over-representation of genes related to developmental processes (11.7% compared to 6.2% in the whole genome) and, in particular, to cell differentiation was observed (Fig. 5A). These genes include for example the α-expansins 7 (AT1G12560) and 18 (AT1G62980), the glycosyl hydrolase 9C1 (AT1G48930), the cellulose synthase like 5 (AT4G15290), the β-galactosidase 6 (AT5G63800) and CER4 (AT4G33790) which encodes an alcohol-forming fatty acyl-CoA reductase involved in cuticular wax biosynthesis. Moreover, this analysis revealed a predominance of genes involved in transport when comparing to the whole genome categorization (15.6% compared
to 4.8 % in the whole genome). Accordingly, genes up-regulated by apo-pyo encode proteins preponderantly located in the plasma membrane, in endomembranes, in the cell wall and also extracellular proteins (Fig. 5B). These four cellular component categories represent 56.8 % of the genes induced by apo-pyo while they represent only 20.6 % of the whole genome. Importantly, this category includes genes related to iron transport including **IRT1** (*AT4G19690*) and **IRT2** (*AT4G19680*; Table S6). Strengthening this data, other genes encoding proteins involved in iron homeostasis were also identified as up-regulated by apo-pyo in roots facing iron deficiency (Table S6). Amongst these, **FRO2** (*AT1G01580*) and the transcription factor **bHLH39** (also named **ORG3**; *AT3G56980*) belonged to the most induced genes. Another transcription factor, **bHLH100** (*AT2G41240*), was highly induced in roots and in shoots. Although to a lower extent, **NAS4** encoding nicotianamine synthase 4, was also found as being up-regulated. We recently showed that the corresponding protein, which catalyses the synthesis of the iron chelator *in planta* transporter nicotianamine, plays a key function in the plant response to iron deficiency (Koen et al., 2014). To check the microarray data, we tested the level of accumulation of four transcripts among the most modulated by apo-pyo in iron-deficient medium by RT-qPCR. The results presented in Fig. S1 were in agreement with the CATMA transcriptomic data. In particular, we noticed a major induction of **bHLH39** and **AT2G38240** (encoding a 2-oxoglutarate and Fe^{2+}-dependent oxygenase superfamily protein) in the shoots and of **FRO2**, **bHLH39** and **IRT1** in the roots in response to apo-pyo. Regarding the genes whose expression was deeply repressed by apo-pyo in roots and shoots of plants grown in iron-deficient condition, an over-representation of genes involved in stress response (18.7 % compared to 6.3 % in the whole genome) and to abiotic or biotic stimulus (16.1 % compared to 5.6 % in the whole genome; Fig. 5A) was observed. For instance, we identified the lipid transfer protein **LTP3** (*AT5G59320*) that is predicted to encode a pathogenesis-related protein (Table S7). As described for up-regulated genes, down-regulated genes encoding extracellular proteins (21.7 % *versus* 4.8 % in the whole genome) and proteins located in the cell wall (4.3 % *versus* 1.3 % in the whole genome) were well represented compared to the data of the whole genome (Fig. 5B). It should be also specified that **AtFER1**, encoding FERRITIN 1 involved in iron sequestration, was found to be strongly down-regulated in roots of iron-deprived plants (Tables S6 and S7). This data further supports the assumption that apo-pyo impacts the iron homeostasis of plants grown in iron-deficient condition.
The functional categorization of the root genes that expression was strongly modulated by apo-pyo treatment (log 2 ratio ≥ + 3 or ≤ - 3; p-value ≤ 0.05) in plants grown in iron-deficient condition was further assessed using AmiGO and Mapman program. This analysis clearly confirmed an incidence of apo-pyo in the expression of genes involved in development and biotic stresses (Fig. S2A). The genes gathered in the biotic stress category encode proteins playing roles in hormone signaling, in cell wall dynamic and in signaling processes (Fig. S2B). To complete this investigation, we performed a more systemic analysis by including root genes regulated by apo-pyo to a lower extent (log 2 ratio ≥ + 1.5 or ≤ -1.5; p-value ≤ 0.05) in plant facing iron-deficiency. We noticed a repression of genes encoding resistance genes (R genes), transcription factors ERF, WRKY and MYB, salicylic acid (SA)-related genes (such as AT5G24210 belonging to the lipase class 3 family protein) and abscisic acid (ABA)-related genes (such as the Lipid Transfer Protein LTP3) (Fig. S2C and Table S7). The observation that genes related to the SA and ABA pathways were repressed prompted us to compare our transcriptomic data to those recorded for hormone perturbations in Genevestigator. The on-line analysis revealed that many of the root genes modulated by apo-pyo in iron-deficient condition were inversely expressed in A. thaliana exposed to SA or ABA (Fig. S3). These data reinforce the possibility that apo-pyo might repress the SA/ABA pathways. We also compared our transcriptomic signature to microarray analyses searching for genes modulated in response to elicitors and biotic stresses. This comparison highlighted that most of the apo-pyo-modulated root genes of plants facing iron deficiency were regulated in an opposite way than in response to the pathogens Phytophthora parasitica, Sclerotinia sclerotiorum and Pseudomonas syringae pv. maculicola (Fig. S4). These results support the hypothesis that apo-pyo could display biological properties related to plant defence responses. Collectively, the transcriptomic analysis revealed that apo-pyo treatment of plants facing iron deficiency impacted the expression of numerous genes. Notably, apo-pyo positively regulated the expression of genes related to development and iron acquisition (uptake/transport). On the opposite, several genes encoding proteins involved in iron sequestration (ferritin) but also in the plant response to biotic stresses were repressed.

3. Perturbations of iron homeostasis induced by apo-pyo

The impact of apo-pyo on the expression level of genes related to iron homeostasis led us to investigate its impact on the plant iron content in our different conditions (see Fig. 1 for the experimental design), 6 h and 3 days after the addition of apo-pyo.
First, we measured the iron content in the nutritive medium. In the Fe 25 medium, a decrease of the iron content occurred between 6 h and 3 days after the treatment, indicating that plants imported part of the iron present in the medium. When apo-pyo was added in the same medium, the concentration of iron did not decrease with time (Fig. 6A). The measurement of iron contents in roots further supported these data (Fig. 6B). Indeed, compared to the Fe 25 treatment, the addition of apo-pyo in the medium containing 25 µM of iron (Fe 25 apo-pyo condition) strongly reduced the root iron concentration at 6 h and 3 days (Fig. 6B). In contrast, the iron content in roots of plants grown in the Fe 25 µM condition without apo-pyo remained almost constant over time. As expected, this first set of data indicates that apo-pyo negatively impacts the dynamic of iron import from the medium by plant roots, probably by direct chelation of iron in the medium. Accordingly, the expression of IRT1 and FRO2 were repressed 3 days after the addition of apo-pyo in the iron containing medium (Table S6). The comparison of the Fe 0 and Fe 0 apo-pyo conditions was also relevant. In the absence of apo-pyo, the level of iron at 6 h in the roots of Fe 0-treated plants was reduced compared to Fe 25-treated plants (Fig. 6B). Therefore, it is likely that plants already perceived the lack of iron in the medium. In comparison, the addition of apo-pyo did not further alter the root iron content of plants grown in the iron-deficient condition. In contrast, at day 3, whereas the iron content of the Fe 0 plants was almost similar to that measured at 6 h, Fe 0 apo-pyo-treated plants showed a strong reduction in the iron content. Taken as a whole, these results indicate that the addition of apo-pyo in the culture medium containing or missing iron triggered a reduction of the root iron content.

Completing this analysis, the iron content in shoots was also measured (Fig. 6C). Six hours after the treatments, the iron content was similar in all conditions. After 3 days, while the apo-pyo treatments induced a strong reduction of the root iron content in culture media containing or not iron (Fig. 6B), the same treatments did not impact the iron concentration in shoots (Fig. 6C). In contrast, the iron content in shoots of plants grown in medium lacking iron without apo-pyo (Fe 0 treatment) slightly but significantly decreased.

To further investigate the incidence of apo-pyo treatment on iron homeostasis, we performed a functional analysis of 6 key iron-related genes found to be modulated by apo-pyo: IRT1, FRO2, FER1, NAS4, bHLH100 and bHLH39/ORG3. As already discussed, with the exception of FER1 which was repressed, the expression of these genes was up-regulated in the roots in the Fe 0 apo-pyo condition. Furthermore, FRO2 and IRT1 expressions were down-regulated in the Fe 25 apo-pyo condition (see above, Table S6). We therefore studied whether corresponding mutants could be affected in their response to apo-pyo in iron-deficient or iron-
sufficient media by measuring their shoot and root masses after 7 days of treatment as
described in Fig. 3B and 3C. The response to apo-pyo was not modified in the *bhlh100* and
*bhlh39/org3* mutants as well as in the *FER1* overexpressor (ov. fer.) whatever the iron content
in the culture medium (Fig. S5A to S5D). In contrast, compared to wild-type (WT), the *irt1*
and *fro2* KO mutants showed an impaired response to apo-pyo in iron-deficient medium in
shoots (Fig. 7A and 7C) and in roots (Fig. 7B and 7D). Indeed, the growth-promoting effect
triggered by apo-pyo in WT was strongly reduced in both mutants in this culture condition,
highlighting the involvement of IRT1 and FRO2 in this process. Regarding NAS4, whereas
the KO *nas4* mutant did not showed a modified response to apo-pyo as compared to WT (Fig.
7E and 7F), the growth promoting effect of the siderophore was significantly more
pronounced in roots of plants overexpressing *NAS4* (35S:NAS4) grown in iron deficiency
(Fig. 7F). Similarly to WT plants, apo-pyo treatment did not affect the shoot and the root
masses of all the mutants and overexpressors tested when grown in iron-sufficient conditions.
Collectively, these data highlight an involvement of IRT1, FRO2 and, to a lower extent, of
*NAS4* in the growth effect of apo-pyo in plants exposed to iron deficiency.

4. Incidence of apo-pyo in SA, ABA, IAA and ethylene synthesis/accumulation

The finding that apo-pyo modulated the expression of genes related to SA and ABA
signalings and increased the growth of plants in medium deprived of iron prompted us to
analyse the concentration of several hormones in our distinct conditions, namely SA, ABA,
indole-3-acetic acid (IAA, the major endogenous auxin) and ethylene. Hormone
measurements were performed 6 h and 3 days after apo-pyo treatment (see Fig. 1 for the
experimental design).

In plants grown in iron-containing medium, at 3 days, we only measured a slight decrease of
SA content by apo-pyo (Fe 25 apo-pyo *versus* Fe 25) (Fig. 8A). Concerning ABA, iron
deficiency induced a clear increase of its concentration at 6 h and 3 days (Fe 0 condition
*versus* Fe 25 condition) (Fig. 8B). At 6 h, the addition of apo-pyo did not influence the ABA
content in iron containing or missing culture media. However, after 3 days, the higher level of
ABA measured in the shoots of plants grown in iron-deficient conditions (Fe 0) was not
observed anymore in plants grown in the same medium but supplied with apo-pyo (Fe 0 apo-
pyo). In this latter condition, the shoot concentration of ABA was similar than those found in
the shoots of Fe 25- and Fe 25 apo-pyo-treated plants. Therefore, apo-pyo appeared to
negatively impact ABA synthesis/accumulation in iron-deficient condition at 3 days, the time
point where apo-pyo begins to rescue the growth of plants facing this deficiency (see Fig. 3).
In the case of IAA, there was no difference after 6 h whatever the treatment. However, after 3 days, iron deficiency induced a reduction of the content of IAA (Fe 25 versus Fe 0 treatments; Fig. 8C). The presence of apo-pyo in Fe 25 or Fe 0 medium had no significant additive effect on the IAA level. In addition, none of the treatments significantly affected the ethylene content of the plantlets (Fig. 8D).

5. Involvement of apo-pyo in the growth/defense balance

We and others recently reported that iron deficiency induces an increased resistance of *A. thaliana* plants against the fungal pathogen *B. cinerea* (Kieu et al., 2012; Koen et al., 2014). The findings that apo-pyo negatively regulated the levels of ABA and the expression of defense-related genes of plants in the iron-deficiency condition let us to check whether the siderophore could impact the plant resistance to this pathogen. For this purpose, leaves of plants exposed to our different conditions (see Fig. 1 for the experimental design) were inoculated by *B. cinerea* at day 3 and the disease symptoms were measured 4 days after the inoculation. As expected and as previously reported, the average of the necrotic lesion areas produced by *B. cinerea* infection was reduced in plants that suffered from iron deficiency compared to plants grown in the Fe 25 medium (22 mm² versus 50 mm²; Fig. 9). Interestingly, the induced resistance conferred by iron deficiency was partly impaired in response to apo-pyo. Indeed, compared to the condition without apo-pyo, plants grown in iron-deficient medium in presence of the siderophore were more sensitive to the fungal infection (necrotic lesion areas about 36 mm²). In contrast, no effect of apo-pyo was observed in plants grown in iron-sufficient condition.

To further understand how apo-pyo impacts the growth and defense of plants grown under iron deficiency, we analyzed the involvement of 4 genes highly modulated by the siderophore and previously shown to display important functions in development and/or defense. These gene include *LTP3*, *CER4*, *AT5G59320* (named *PLTP* in this paper) encoding a protein involved in lipid transport and binding and whose transcripts were shown to be induced in leaves of *A. thaliana* plants subjected to a combination of drought and heat stress (Rizhsky et al., 2004) and *AT3G21460* (named GRX in this paper) which encodes a glutaredoxin family protein involved in cell redox homeostasis (Finkemeier et al., 2005). The growth-promoting effects of apo-pyo observed in plants facing iron deficiency was not altered in the mutants impaired in the expression of the genes of interest (Fig. S5A, S5B, S5E and S5F).

We completed this analysis by investigating whether apo-pyo could impact the balance between growth and defense as the siderophore had a positive effect on the growth but a
negative effect on the resistance to *B. cinerea* in plants cultivated in iron deficiency. Recently, it has been demonstrated that this trade-off can be based on cross-talks operating between growth- and immune-related signaling pathways (Bai et al., 2012; Fan et al., 2014; Malinovsky et al., 2014). Notably, it has been shown that such cross-talks could involve 2 transcription factors namely BZR1 and HBI1, the second being a partner of the tripartite module PRE1-IBH1-HBI1 that regulates cellular elongation upstream from external or endogenous signals as brassinosteroids (BR) or temperature (Fan et al., 2014). IBH1 interacts with and inhibits HBI1 which is a positive regulator of cellular elongation. In particular, HBI1 activates *ATEXPA8* and *ATEXPA1*, 2 genes encoding expansins (cell wall-loosening enzymes) by direct binding to their promoters. The effects of IBH1 on HBI1 are inhibited by PRE1 which sequestrates IBH1. We first checked the modulation of these different genes in the microarray experiment (Table S8). It appeared that *HBI1* and *PRE1* were highly up-regulated by apo-pyo in the shoots of Fe 0 apo-pyo- versus Fe 0-treated plants while the inhibitor IBH1 was slightly down-regulated. We also noticed an induction of the expression of *ATEXP1* and *ATEXP8*. Moreover, other genes encoding expansins were also activated in response to apo-pyo in roots and/or shoots and could be involved in the siderophore-induced promotion of growth in iron deficiency.

As no *HBI1*-deficient mutant is available (Malinovsky et al., 2014), we tested the phenotype of *HBI1*-ox plants which over-express *HBI1* and *HBI1*(L214E)-ox plants that display dominant-negative effect on *HBI1* in our different conditions (see Fig. 1 for the experimental design). In the absence of apo-pyo, *HBI1*-ox plants had higher growth that WT plants in Fe 25 medium (Fig. 10). However, in response to iron deprivation, the shoot (Fig. 10A) and root (Fig. 10B) masses of *HBI1*-ox plants were similar to those of WT plants. Finally, the growth-promoting effects of apo-pyo observed in WT plants facing iron deficiency were not statistically different in *HBI1*-ox and *HBI1*(L214E)-ox plants.

6. Comparison of apo-pyo effects to those induced by C7R12 bacterial inoculation

Four week-old plants were subjected to apo-pyo treatment as described in Fig. 1 or inoculated with the wild-type *P. fluorescens* strain C7R12 or with the mutant strain PL1 in iron-deficient or iron-sufficient medium. PL1 is a pyoverdine minus C7R12 mutant obtained by random insertion of the transposon Tn5 in the pyoverdine synthetase gene *pvsB* (Mirleau et al., 2000). PL1 is unable to produce pyoverdine but produces ornicorrgatine, another siderophore. First, we compared the macroscopic phenotypic effects of the purified siderophore to those of the bacterial inoculation with C7R12 or PL1 strains. As described for apo-pyo (Fig. 3), no
effect of C7R12 nor PL1 was visible in iron-sufficient medium after 7 (Fig. S7A) or 14 days of treatment (Fig. S7B). Indeed, root and shoot weights of the inoculated plants were equivalent to those of non-inoculated plants (Fig. 11A and 11B). Importantly, in contrast with the treatment with purified apo-pyo, in iron-deficient conditions, the C7R12 strain producing apo-pyo did not partially restore the growth of plants (Fig. S7, Fig. 11A and 11B). However, compared to plants grown in iron-deficient medium without apo-pyo treatment (Fe 0 treated plants), the C7R12 strain triggered a reduction of the violet pigmentation after 14 days of treatment in the iron-deprived medium (Fig. S7B). Accordingly, the production of anthocyanins measured in C7R12-treated plants facing iron deficiency was intermediate between those measured in Fe 0- and Fe 0 apo-pyo-treated plants (Fig. 11C). Highlighting the putative involvement of apo-pyo in this process, the reduction of the anthocyanin level observed in C7R12-treated plants was not found when plants suffering from iron deficiency were inoculated with the PL1 strain. Indeed, PL1-treated plants displayed an anthocyanin concentration similar to that of Fe 0-treated plants (Fig. 11C).

We also compared the sensitivity to B. cinerea of the plants treated with apo-pyo to those inoculated with the C7R12 or PL1 strains. No effect of both strains was observed in the Fe 25 medium as described previously for apo-pyo (Fig. 12). In the medium lacking iron, C7R12-inoculated plants (Fe 0 C7R12) were more sensitive to the pathogen than Fe 0-treated plants, their higher sensitivity being intermediate between those of Fe 0 apo-pyo treated-plants and Fe 0 PL1-treated plants. These data strongly suggest that the production of apo-pyo by the wild-type strain was responsible for the higher sensitivity of the plants to B. cinerea in iron-deficient medium.

DISCUSSION

Most of the studies dealing with the effects of bacterial siderophores on plants were achieved in non-limiting iron nutrition media and/or using ferri-siderophores (for instance see Vansuyt et al., 2007; Aznar et al., 2014). Here, we analysed the biological effects of a non-chelated siderophore, apo-pyo, on plants that were maintained in a medium depleted or not of iron. This enabled to mimic a situation in which the two partners, bacterial strain and host-plant, are submitted to iron deficiency and make attempt to acquire iron from the rhizosphere. As a matter of fact, siderophores are known to be only produced by bacteria in iron stress conditions (Meyer and Abdallah, 1978) as the rhizosphere environment in which pyoverdine synthesis was evidenced using a reporter gene (Loper et al., 1997; Duijff et al., 1999).
We first showed that apo-pyo was effectively taken up by roots when plants were cultivated in hydroponic solution. This phenomenon was previously shown to occur in plants cultivated *in vitro* and treated by ferri-pyo in a medium deprived of iron (Vansuyt et al., 2007). Moreover, using $^{15}$N-apo-pyo, we were also able to detect $^{15}$N in the shoots. The fact that apo-pyo was not detected by ELISA in the shoots could be explained by the less sensitivity of this technique compared to $^{15}$N-detection by mass spectrometry or, eventually, by a degradation or a chemical modification that would impair its recognition by antibodies as previously suggested by Vansuyt et al. (2007). In both cases, this result indicates that apo-pyo or a derivative is a systemic compound. Moreover, the quantity of apo-pyo assimilated by plants did not differ whether the plants were pre-cultivated 24 h in presence or not of iron. This observation indicates that plants may not adapt the rate of apo-pyo assimilation according to the availability of iron in the rhizosphere.

Apo-pyo had a striking macroscopic effect on plants facing iron deficiency. Compared to non-treated plants grown without iron, it induced a remarkable promotion of growth, together with a reduction of anthocyanin accumulation. We checked that the iron-free medium was still deprived of iron once apo-pyo was added, thus ruling out the possibility that the growth phenotype observed in the presence of the siderophore was due to an iron contamination. A promotion of growth was already observed in Vansuyt et al. (2007) in iron-deficient medium following the addition of ferri-pyo to the roots of *A. thaliana*. This promotion was significantly higher with ferri-pyo than with Fe-EDTA and was associated with a higher increase in the iron content of the plants.

To strengthen these data, we performed similar experiments using the wild type C7R12 strain and the corresponding PL1 strain impaired in apo-pyo production. Similarly to purified apo-pyo treatment, in the iron deficiency conditions, the C7R12 strain triggered a reduction of anthocyanin accumulation when compared to Fe 0 treated plants. This phenomenon was due to apo-pyo production since it was not visible in response to PL1 inoculation. However, the promotion of growth induced by the purified apo-pyo in iron-deficient conditions was not observed in Fe 0 C7R12-inoculated plants. This discrepancy may be explained by different factors. First, it is conceivable that the concentration of the apo-pyo released by the bacteria may be different from that of the purified apo-pyo (25 μM). Secondly, beside apo-pyo, other compounds produced by the bacteria and recognized by the plant such as lipo- or exo-polysaccharides, as well as molecules secreted into plant host cells may also account for the effects of C7R12, thus making the situation much more complex. Indeed, it has been shown that numerous plant-beneficial pseudomonads as *P. fluorescens* C7R12 harbour type 3
secretion system (T3SS)-like genes related to the Hrp1 (Hypersensitive response and pathogenicity 1)-T3SS family (Mazurier et al, 2004). This T3SS contributes to the plant-bacteria interactions and to the bacterial effects in plants. Involvement and role of this system in *A. thaliana*/C7R12 bacteria interaction remain to be determined.

To investigate the mechanisms underlying apo-pyo effects, we choose to perform a large-scale transcriptomic analysis. It appeared that apo-pyo modulated the expression of around 2100 genes (log 2 ratio ≥ +1.5 or ≤ -1.5; p-value ≤ 0.05). The number of genes modulated by apo-pyo and their induction or repression status clearly depended on the tissue and/or the presence or not of iron in the hydroponic solution. First, root genes rather than shoot genes were preferentially modulated. This result was expected as apo-pyo was applied in the nutrient solution, directly in contact with the roots, and once assimilated by plants accumulated preferentially in roots rather than in shoots. Secondly, a great part of the apo-pyo-dependent genes was modulated in iron-deficiency rather than in iron-sufficient condition. Therefore, the effect of apo-pyo on gene expression appeared to be dependent on the plant iron status. Furthermore, the higher number of apo-pyo-modulated genes noticed in plants grown in the iron-deficient medium corroborates the strong phenotypic effect triggered by the siderophore in this condition.

A focus on the apo-pyo-regulated genes in plants grown in iron free medium showed that the most induced genes encoded proteins involved in development and iron acquisition processes while the most repressed were related to iron sequestration and defense responses. Interestingly, Aznar et al. (2014) recently reported data of a transcriptomic analysis performed on *A. thaliana* plantlets cultivated in iron-sufficient conditions and in which the apo-form of the siderophore deferrioxamine (DFO, 1 mM) was infiltrated in leaves. The major categories of genes modulated by DFO in leaves were related to biotic and abiotic stresses including immune response, whereas in roots the DFO-target genes were associated to heavy-metal homeostasis. Importantly, no effect on gene modulation was observed when Fe-DFO complexes were used instead of iron-free DFO and the physiological responses induced by DFO were similarly observed in response to a synthetic iron chelator. Therefore, the effects of DFO were due, or partly due, to its iron-chelation ability. Inevitably, these data raise the question of the incidence of apo-pyo on the plant iron homeostasis and whether its effects could be also related to its chelating properties. The transcriptomic analysis as well as the quantification of the iron content in apo-pyo-treated plants provided first answers. In plants grown in iron-containing medium, the addition of apo-pyo did not affect the shoot iron content but triggered a strong reduction of the root iron concentration accompanied by a
repression of IRT1 and FRO2 expression. Furthermore, many of the genes regulated by apo-pyo in roots were also found to be modulated by iron deficiency in the roots of A. thaliana in the study of Schuler et al. (2011) (listed in Table S9) and, using Genevestigator, we identified a profile closed to those of two iron deficiency treatments (Fig. S6). Therefore, apo-pyo triggered an iron deficiency-like response in plants grown in iron-sufficient condition. As expected, we provided evidence that this process was mainly due to the chelation by apo-pyo of the iron present in the culture medium. Intriguingly, the resulting reduction of iron availability did not impact the growth of the plants. This observation suggests that although the apo-pyo treatment impacted the root iron content, the iron concentration of plants grown in iron-containing medium was probably sufficient for ensuring normal growth. This assumption does not exclude the possibility that part of the siderophore imported by the plants could be internalized in its iron-chelated structure and contribute to iron assimilation. If it is the case, this mechanism poorly accounts for the plant iron nutrition.

In plants facing iron deficiency, apo-pyo also triggered a strong reduction of the root iron content compared to non-treated plants grown in iron free medium. However, the reduction of shoot iron concentration observed in the latter was less pronounced in the apo-pyo-treated plants. It is striking that, in plants grown in iron-deficient condition, apo-pyo promoted an important induction of genes related to iron assimilation such as IRT1, FRO2 and ORG3 as well as a down regulation of AtFER1. These data deeply contrast with those obtained in apo-pyo-treated plants grown in iron-sufficient conditions and highlight that in plant facing iron-deficiency apo-pyo strongly promotes the iron uptake machinery. Furthermore, in addition to the iron uptake process, apo-pyo also induced a strong expression of genes encoding proteins related to in planta iron redistribution such as NAS4 and OPT3 (see Table S6; Koen et al., 2014; Zhai et al., 2014). As plants were grown in iron free condition, it is reasonable to assume that apo-pyo provided in the culture could be imported by plants in its iron-free structure and, once internalized in roots, could chelate cellular iron. Accordingly, due to their high affinity for iron, bacterial siderophores including pyoverdine are able to efficiently compete with host iron-binding proteins for iron acquisition (Saha et al., 2013). This process could account for the ability of apo-pyo to induce the overexpression of the plant iron import system. Whatever the scenario, we provide evidence that the induction of the iron import machinery contributes to the growth promoting effects of apo-pyo in plants facing iron deficiency. Indeed, the positive effect of apo-pyo on growth was severely impaired in the mutants deficient in IRT1 and FRO2 expression. Furthermore, the positive effect of apo-pyo on growth was further induced in the 35S:NAS4 plants overproducing NA, a non-
proteinogenic amino acid that contributes to iron long distance circulation in phloem and transfer within the cells and which is required for the proper establishment of the plant response to iron deficiency (Koen et al., 2014).

The analysis of the content of ABA in plants exposed to apo-pyo also supports the findings that apo-pyo had a major impact on iron homeostasis. First, the hormone accumulated in the shoots of plants suffering from iron deficiency in presence or not of apo-pyo within 6 h. Secondly, after 3 days of apo-pyo treatment, plants grown in iron free medium showed a drastic reduction of their shoot ABA level compared to non-treated plants cultivated in the same medium. Therefore, the production of ABA appeared to be more transient in plants exposed to apo-pyo. Accordingly, the transcriptomic analysis revealed that in the iron free condition, apo-pyo repressed the expression of root genes related to ABA signaling. Our data should be discussed in the light of Lei et al. (2014) investigation. These authors reported that Fe deficiency induced a rapid ABA accumulation (within 6 h) in the roots of *A. thaliana*. In this study, ABA was shown to contribute to alleviate iron deficiency by promoting root iron reutilization and transport from the roots to the shoots. This process involved the ABA-dependent regulation of genes encoding proteins related to the reutilization of the iron stored in the vacuoles as *AtNRAMP3* and to long-distance transport of Fe including *AtFRD3*, *AtYSL2* and *AtNAS1*. In our study, the observations that, in iron deficiency, the apo-pyo-treated plants displayed an induced expression of root genes related to iron in planta transport/redistribution, suggest that the mechanism described by Lei et al. (2014) could operate efficiently and, therefore, more transiently, in response to the siderophore. However, none of the ABA-dependent genes described by these authors were found to be modulated by apo-pyo after 3 days of treatment, thus minimizing this possibility.

Recent investigations have reported that the plant iron status conditions its resistance against microorganisms. Notably, it has been found that iron deficiency activates defense-related processes and confers resistance against microbial pathogens including *Dickeya dadantii* and *Botrytis cinerea* (Kieu et al., 2012; Koen et al., 2014). Several studies also demonstrated that siderophores produced by microorganisms trigger an immune response that could be linked to their capacity to chelate iron from the host (Aznar and Dellagi, 2015; Aznar et al., 2015). In the same vein, we recently demonstrated that β-aminobutyric acid (BABA), a priming agent that confers enhanced resistance to numerous pathogens, is a powerful iron chelator triggering a transient iron deficiency in plants. This process could bring the plant to a defense-ready state contributing to an efficient resistance (Koen et al., 2014). Further strengthening the concept that plant immunity is tightly linked to iron homeostasis, several genes including
those encoding the transcription factor MYB72 (AT1G56160) and the β-glucosidase BGLU42 (AT5G36890) were shown to function in the cross-talk of root signaling pathways regulating rhizobacteria-mediated ISR and the plant response to iron deficiency (Zamioudis et al., 2014 and 2015). In the present investigation, in accordance with the studies listed above, we confirmed that plants cultivated in iron deficiency displayed a higher resistance to *B. cinerea*. This higher resistance was reduced by the apo-pyo treatment but also by a C7R12 bacterial inoculation. In response to apo-pyo, this negative effect was correlated to a general down-regulation of genes encoding proteins involved in immunity with few exceptions. In particular, the expression of BGLU42 required for rhizobia-induced ISR was slightly induced in apo-pyo-treated plants (Table S6). Several works demonstrated that pyoverdines act as determinants in ISR against numerous pathogens. The induction of ISR is not systematic and depends on the considered pathosystem and the type of pyoverdine. For instance, some pyoverdines as those of C7R12 induce resistance against the rice blast but some do not. De Vleesschauwer and Höfte (2008) suggested that these differences can be explained by the induction of iron deficiency by certain pyoverdines. In our case, despite the induction of an iron-deficiency like response in the Fe 25 medium, apo-pyo did not induce ISR in *A. thaliana* against *B. cinerea*. Other factors are probably involved in the induction of ISR.

We observed that pyoverdine did not favour the resistance against *B. cinerea* but rather partially impaired it in plants grown in iron-deficient condition. The interpretation of this process should take into account the drastic growth-promoting activity of apo-pyo. Indeed, the antagonistic effect of apo-pyo on growth and resistance observed in plants facing iron deficiency strongly suggests that the siderophore impacts the trade-off between growth and immunity. As recently highlighted in several studies, cross-talks between growth and immune signalings operate in plants. According to the physiological contexts, notably pathogen challenge or light conditions, these cross-talks prioritize one process over the second one (reviewed in Lozano-Durán & Zipfel, 2015). Clearly, our data indicate that, in plants grown in iron-deficient conditions and in response to apo-pyo, growth is prioritized over immunity. Further supporting this assumption, the microarray analysis revealed that apo-pyo modulates the expression of the PRE1-IBH1-HBI1 module in shoots of plants facing iron deficiency. As discussed in the Results section, HBI1 is a key node for the cross-talk between growth and immunity and, once activated, negatively regulates defense responses while prioritizing growth (Fan et al., 2014; Malinovsky et al., 2014). HBI1 effects are antagonized by IBH1. In turn, through its binding to HBI1, PRE1 prevents its inhibitory effect (Bai et al., 2012). Supporting a role of HBI1 in the mediation of the trade-off between growth and immunity, its
overexpression was shown to trigger the repression of the expression of genes involved in immunity and to enhance susceptibility of the plants to the bacterium *Pseudomonas syringae* (Fan et al., 2014; Malinovsky et al., 2014). We found that both *HBI1* and *PRE1* expression in shoots were strongly up-regulated in response to apo-pyo whereas the expression of *IBH1* was slightly repressed. Therefore, these expression profiles reflect a physiological situation in which the balance between growth and immunity is weighted in favour of growth. To strengthen this possibility, we checked whether the growth promoting effect of apo-pyo in plants grown in iron-deficient condition was enhanced in the *HBI1* overexpressing line (*HBI1*-ox). We did not observe such effect. This data could be explained by the fact that the level of *HBI1* expression induced by apo-pyo in WT plants was particularly high.
CONCLUSION

To conclude, the present investigation provides evidence that the effects of pyoverdine depend on iron availability and, consequently, on the plant iron status. Furthermore, it highlights that in plants facing iron deficiency, the apo-siderophore modulates the trade-off between growth and immunity in favour of growth, this mechanism being dependent on the expression of the iron-uptake related genes *IRT1* and *FRO2*. More generally, this work indicates that microorganism-derived siderophores impact the cross-regulatory interactions operating on host plants between iron homeostasis, immune function and growth.
MATERIALS AND METHODS

Plant material and growth conditions

*Arabidopsis thaliana* Columbia-0 (Col-0) ecotype was used as wild-type (WT) control for phenotype comparisons for all mutants and transgenic lines described in this study. The T-DNA mutants *bhlh100* (SALK_074568C), *bhlh39* (SALK_025676C), *cer4* (SALK_000575C), *grx* (SALK_001139C), *ltp3* (SALK_095248C), and *pltp* (SALK_063856) were obtained from the Nottingham Arabidopsis Stock Center (NASC, Nottingham, United Kingdom). Homozygosity for the mutant alleles was checked using specific primers (Table S10). The following lines were described previously: *irt1* (Vert et al., 2002), *fro2* (also named *frd1-1*; Robinson et al., 1999), *ov. fer.* (OV ferritin; Vansuyt et al., 2007), *HBI1-ox* and *HBI1*(L214E)-ox (Malinovsky et al., 2014), 35S:*NAS4* and *nas4* (Koen et al., 2013).

Seeds were surface sterilized by immersion in a solution containing 1.2 % bleach and 50 % EtOH for 3 min, rinsed three times with 100 % EtOH, air-dried and placed for 3 days at 4°C in the dark in 0.15 % agar. Then, they were sown in seed holders filled with 0.75 % agar and put in a cover placed above the nutrient solution container (Araponics, Liege, Belgium). The nutritive medium named Fe 25 nutrient solution (0.25 mM Ca(NO₃)₂, 0.5 mM KNO₃, 1 mM KH₂PO₄, 1 mM MgSO₄, 50 μM H₂BO₃, 19 μM MnCl₂, 10 μM ZnCl₂, 1 μM CuSO₄, 0.02 μM Na₂MoO₄, 25 μM Fe-Na-EDTA) was changed twice a week. Plants were allowed to grow in a climate-controlled growth chamber for four or five weeks before the treatments in the following conditions: 10 h day (200 μE.m⁻².s⁻¹ light intensity, 20°C) / 14 h night (18°C) with 70 % relative humidity.

Chemicals

All chemicals were purchased from Sigma-Aldrich (Saint-Louis, USA) unless stated.

Production and purification of apo-pyo and ¹⁵N-apo-pyo

*Pseudomonas fluorescens* C7R12 has been isolated from a soil naturally suppressive to soilborne disease and is an efficient biocontrol agent against pathogenic *Fusarium oxysporum* (Lemanceau et al., 1991). *P. fluorescens* was grown on King’s B (KB; King et al., 1954) agar plate for two days at 25°C. Several colonies were sowed in succinate medium and incubated with shaking (180 rpm) for one day at 25°C (Meyer and Abdallah, 1978). Few millilitres of this culture were used to inoculate the main culture (650 mL in 3 L Erlenmeyer) that was incubated for four days in the same conditions as described above. After incubation, bacterial...
cells were removed by two centrifugations at 4,800 g for 30 min (4°C). The pool of resulting
supernatants was adjusted at pH 6 with 6 N HCl and then was mixed with an
Amberlite®XAD-4 resin (Carson et al., 2000), 300 mL of resin being mixed under agitation
with 3.9 mL of the supernatant for 4 h. After loading, the resin was washed with mQ water
and apo-pyo was eluted from the column overnight with 100 % methanol at 4°C. The eluate
was concentrated with a rotary evaporator in order to obtain a 2-3 mL solution. The obtained
solution was adsorbed on a Sep-Pak C18 Vac 6 cc cartridge containing 500 mg of
octadecylsilane (Waters Co. Ltd., Bedford, MA, USA; Nagata et al., 2013). After loading, the
column was washed with 0.1 M EDTA, followed by pH 4.0 acidified water (1 % formic acid)
and apo-pyo was eluted with aqueous 80 % methanol and finally concentrated with a rotary
evaporator and lyophilized prior to storage of the powder at -20°C. The same protocol was
used to produce 15N-pyoverdine with supply of (15NH₄)₂SO₄ 98 % to the succinate medium.

Apo-pyo treatments

After 4 weeks of culture (or 5 weeks for infection experiments), plant roots were rinsed using
Fe 0 nutrient solution (hydroponic solution described above without Fe-Na-EDTA) and plants
were separated in 4 batches corresponding to 4 treatments (Fig. 1):
- Fe 25: pretreatment for 1 day in Fe 25 nutrient solution and treatment for 7 days in Fe 25
  nutrient solution,
- Fe 25 apo-pyo: pretreatment for 1 day in Fe 25 nutrient solution and treatment for 7 days in
  Fe 25 nutrient solution supplemented with 25 µM of purified apo-pyo.
- Fe 0: pretreatment for 1 day in Fe 0 nutrient solution and treatment for 7 days in Fe 0
  nutrient solution,
- Fe 0 apo-pyo: pretreatment for 1 day in Fe 0 nutrient solution and treatment for 7 days in Fe
  0 nutrient solution supplemented with 25 µM of purified apo-pyo.

Bacterial inoculations

Wild-type C7R12 and Tn5 mutant PL1 (Mirleau et al., 2000) were grown on agar slant tubes
containing KB medium for 72 h at 25°C. Bacterial inoculants were produced on KB agar
plates and incubated at 25°C for 48 h. Bacteria were scraped from the medium and suspended
in sterile distilled water. The bacterial density of the suspensions was determined using a
calibration curve assessed by turbidity (λ = 600 nm). A 1.4-mL inoculum of C7R12 or PL1
was introduced in the plant nutrient medium to obtain a bacterial density of 10⁶ colony
forming units (CFU) per mL of solution.
ELISA procedure for the detection of pyoverdine in roots

Primary antibodies production was described in Vansuyt et al. (2007). Roots of 3 plants per condition of treatment were washed 10 min with mQ water and 2 times 10 min with the following solution: EDTA 5 mM, KCl 1 mM, Na$_2$S$_2$O$_4$ 5 mM, CaSO$_4$ 0.5 mM pH 6 and finally 10 min with mQ water. Roots were frozen and grounded with pestle and mortar in liquid nitrogen. Proteins were extracted with 15 mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$, 100 mM NaCl, 10% glycerol, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 10 mM PMSF and a protease inhibitor cocktail (UltraCruz® Protease Inhibitor Cocktail Tablet, Santa Cruz Biotechnology Dallas, Texas, U.S.A.) following the manufacturer’s instructions. After centrifugation of the samples (14000 g, 15 min, 4°C), protein concentration of the supernatants was estimated according to the procedure of Bradford (1976). Root protein extracts were diluted to obtain 10 µg of proteins in 100 µl of TBS which were loaded in each well of a microtitration plate (NUNC Maxisorp®, San Diego, CA, U.S.A.). These extracts were coated directly overnight at 4°C. After 3 washes of 5 min with 200 µL of TBS-Tween 20 (0.1 %; v/v), each well was saturated with 100 µL of TBS-Tween 20 with 5 % milk (v/v) for 1 h. Antibodies anti-pyoverdine were diluted 100-fold in TBS-Tween 20 with 5 % milk (v/v) and 100 µl per well were added in each well for 2 h. After another 3 washes of 5 min with 200 µL of TBS-Tween 20 (0.1 %; v/v), anti-rabbit secondary antibodies coupled to alkaline phosphatase were diluted 30,000-fold in 50 mM Na$_2$CO$_3$ pH 9.6 and 100 µl per well were added for 1 h. Three washes of 5 min with 200 µL of TBS were achieved and 100 µL of 1 mg.mL$^{-1}$ p-nitrophenyl phosphate ($p$NPP) dissolved in 10 % diethanolamine HCl pH 9.8, 0.5 mM MgCl$_2$ were added in each well. Absorbance was measured at 405 nm for 18 h in a microplate reader (Thermomax, Molecular Devices, Menlo Park, CA, U.S.A.).

Detection of $^{15}$N-apo-pyo

After the treatments, roots or shoots of 4 plants were pooled for each condition. Roots were washed as described in ELISA procedure. Root or shoot tissues were dried at 60°C for 2 days and grounded with pestle and mortar. $^{15}$N was detected and quantified in the tissues by the stable Isotope Analytical platform of B&PMP (Biochimie et Physiologie Moléculaire des Plantes, Montpellier, France).

RNA preparation for transcriptomic analysis
For 3 independent experiments, root and shoot tissues were collected separately after the
treatments and immediately frozen in liquid nitrogen and grounded with a TissueRuptor
(QIAGEN, Venlo, The Netherlands) after addition of 175 µL of RNA lysis buffer (Promega
Corp., Madison, WI, U.S.A.) per 30 mg of tissues. Total RNA extraction was carried out
using the SV Total RNA Isolation System (Promega) according to the manufacturer’s
instructions. RNAs were solubilised in 100 µL RNAse free water. Their concentration and
purity were determined using a NanoDrop 2000 (Thermo Scientific Inc., Madison, WI, USA).
For each experiment, RNA samples from 4 plants per condition were pooled in order to obtain
20 µL of RNAs at 220 ng.µL⁻¹. RNA Integrity Numbers (RINs) were determined with the
2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and considered sufficient
for microarray if comprised between 8.5 and 9.8 for the shoot RNAs and between 7 and 7.8
for the root RNAs.

Microarray experiment
Microarray analysis was carried out at the Institute of Plant Sciences Paris-Saclay (IPS2,
Evry, France), using the CATMA version 6.2 array using the CATMAv6.2 array based on
Roche-NimbleGen technology. A single high density CATMAv6.2 microarray slide contains
twelve chambers, each containing 219,684 primers representing all the Arabidopsis thaliana
genes: 30,834 probes corresponding to CDS TAIRv8 annotation (including 476 probes of
mitochondrial and chloroplast genes) + 1289 probes corresponding to EUGENE software
predictions. Moreover, it included 5352 probes corresponding to repeat elements, 658 probes
for miRNA/MIR, 342 probes for other RNAs (rRNA, tRNA, snRNA, soRNA) and finally 36
controls. Each long primer is triplicate in each chamber for robust analysis and in both strand.
For each comparison, one technical replicate with fluorochrome reversal was performed for
each biological replicate (i.e. four hybridizations per comparison). The labeling of cRNAs
with Cy3-dUTP or Cy5-dUTP (Perkin-Elmer-NEN Life Science Products) was performed as
described in Lurin et al. (2004). The hybridization and washing were performed according to
NimbleGen Arrays User’s Guide v5.1 instructions. Two micron scanning was performed with
InnoScan900 scanner (InnopsysR, Carbonne, France) and raw data were extracted using
MapixR software (InnopsysR, Carbonne, France).

Statistical Analysis of Microarray Data
For each array, the raw data comprised the logarithm of median feature pixel intensity at
wavelengths 635 nm (red) and 532 nm (green). For each array, a global intensity-dependent
normalization using the loess procedure (Yang et al., 2002) was performed to correct the dye bias. The differential analysis is based on the log-ratios averaging over the duplicate probes and over the technical replicates. Hence, the numbers of available data for each gene equal the number of biological replicates and are used to calculate the moderated t-test (Smyth, 2004). Under the null hypothesis, no evidence that the specific variances vary between probes is highlighted by Limma and consequently the moderated t-statistic is assumed to follow a standard normal distribution. To control the false discovery rate, adjusted p-values found using the optimized FDR approach of Storey and Tibshirani (2003) were calculated. We considered as being differentially expressed the probes with an adjusted p-value ≤ 0.05. Analysis was done with the R software. The function SqueezeVar of the library limma has been used to smooth the specific variances by computing empirical Bayes posterior means. The library kerfdr has been used to calculate the adjusted p-values.

**Microarray data deposition**

Microarray data from this article were deposited at Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/), accession no. GSE71163, and at CATdb (http://urgv.evry.inra.fr/CATdb/; Project: INRA13-03_pyo) according to the “Minimum Information About a Microarray Experiment” standards.

**RT-qPCR analyses**

RNA samples were reverse-transcribed using ImpromII™ Reverse Transcriptase kit (Promega) with a mix of anchored oligo(dT)17 and random hexamers primers according to the manufacturer’s specifications. The resulting cDNAs were subjected to a 20-fold dilution with water and 5 µl of each cDNA sample was assayed by qPCR in a LightCycler (ViiA™ 7 Real-Time PCR System, Thermo Fischer) using GoTaq® qPCR Master Mix (Promega) according to the manufacturer’s instructions. Expression levels were calculated relatively to the housekeeping genes AT5G08290 (encoding the mitosis protein YLS8) and AT4G26410 (encoding an uncharacterised conserved protein) using the relative standard curve method. These 2 genes were shown to be among the 5 most reliable reference genes for normalization purposes in *A. thaliana* Col-0 (Wang et al., 2014). For each sample, target quantity of the gene of interest was determined by interpolating the value from the standard curve made from a cDNA pool which enables to take into consideration the efficiency of amplification. The
value was then divided by the target quantity of the housekeeping gene. For a list of primers used for RT-qPCR, see Table S11.

**Determination of Fe concentrations**

After the treatments, roots or shoots of 4 plants were pooled for each condition. Roots were washed 10 min with mQ water, 2 times 10 min with the following solution: EDTA 5 mM, KCl 1 mM, Na₂S₂O₄ 5 mM, CaSO₄ 0.5 mM pH 6 and finally 10 min with mQ water. Root or shoot tissues were dried at 60°C for 2 days. Mineralization of the samples was achieved in Pyrex tubes previously washed 3 h with 0.1 N HCl, 1 h with 5 mM EDTA and rinsed 3 times with mQ water. Once dried, samples were grounded and solubilised in 300 µL of ultra-pure nitric acid (67 % Normaton for trace analysis; VWR BDH Prolabo, Pennsylvanie, U.S.A.). After heating of the samples (1 h at 80°C and 1 h at 100°C), 100 µL of ultra-pure nitric acid and 200 µL H₂O₂ were added in the tubes to obtain a 10 mL solution in mQ water. The concentrations of Fe were determined by inductively coupled plasma-atomic emission spectroscopy (ICP-AES; Vista Pro, Varian, Palo Alto, CA, U.S.A.) by Welience (Pôle de Chimie Moléculaire, Université De Bourgogne, Dijon, France).

**Measurements of anthocyanins**

Shoots from 3 plants per treatment were harvested and immediately frozen in liquid nitrogen. After grounding, anthocyanin content was determined using the protocol described by Teng et al. (2005).

**ABA, SA, JA and IAA content analysis**

Shoots from 5 plants per treatment were harvested, dried and grinded. For each sample, 10 mg of freeze-dried powder were extracted with 0.8 mL of acetone/water/acetic acid (80/19/1 v:v:v). Abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), and indole-3-acetic acid (IAA) stable labelled isotopes used as internal standards were prepared as described in Le Roux et al. (2014). Two ng of each standard was added to the sample. The extract was vigorously shaken for 1 min, sonicated for 1 min at 25 Hz, shaken for 10 minutes at 4°C in a Thermomixer (Eppendorf®), and then centrifuged (8,000 g, 4°C, 10 min). The supernatants were collected, and the pellets were re-extracted twice with 0.4 mL of the same extraction solution, then vigorously shaken (1 min) and sonicated (1 min; 25 Hz). After the centrifugations, the three supernatants were pooled and dried (final volume of 1.6 mL).
Each dry extract was dissolved in 140 µL of acetonitrile/water (50/50; v/v), filtered, and analyzed using a Waters Acquity ultra performance liquid chromatograph coupled to a Waters Xevo Triple quadrupole mass spectrometer TQS (UPLC-ESI-MS/MS). The compounds were separated on a reverse-phase column (Uptisphere C18 UP3HDO, 100*2.1 mm*3µm particle size; Interchim, France) using a flow rate of 0.4 mL.min⁻¹ and a binary gradient: (A) acetic acid 0.1 % in water (v/v) and (B) acetonitrile with 0.1 % acetic acid. The following binary gradient was used (t, % A): (0 min., 98 %), (3 min., 70 %), (7.5 min., 50 %), (8.5 min., 5 %), (9.6 min., 0 %), (13.2 min., 98 %), (15.7 min., 98 %). Mass spectrometry was conducted in electrospray and Multiple Reaction Monitoring scanning mode (MRM mode), in positive mode for IAA and in negative ion mode the other hormones. Relevant instrumental parameters were set as follows: capillary 1.5 kV (negative mode), source block and desolvation gas temperatures 130°C and 500°C, respectively. Nitrogen was used to assist the cone and desolvation (150 L·h⁻¹ and 800 L·h⁻¹, respectively), argon was used as the collision gas at a flow of 0.18 mL/min. The parameters used for MRM quantification of the different hormones are described in Leroux et al. (2014) for the other hormones.

Samples were reconstituted in 140 µL of 50/50 acetonitrile/H₂O (v/v) per mL of injected volume. The limit of detection (LOD) and limit of quantification (LOQ) were extrapolated for each hormone from calibration curves and samples using Quantify module of MassLynx software, version 4.1.

**Ethylene content analysis**

Plants were placed in 30 mL sealed vials containing 10 mL growth medium with the different treatments as described in Fig. 1. Each plantlet weight during the incubation time was on average 125 +/- 6 mg (over 3 biological replicates), and all plants were surrounded by the same headspace volume. Incubation time was set for 6 h or 3 days. After incubation, one mL of headspace gas was sampled and analyzed by gas chromatography (2 m x 3 mm 80/100 alumina column, injector at 110°C, N₂ vector gas in an isocratic oven temperature at 70°C, FID detector at 250°C).

**Plant inoculation with *B. cinerea***

*B. cinerea* BMM strain was grown on 39 g.L⁻¹ Potato Dextrose Agar plates (PDA; BD Biosciences, San Jose, USA) at 23°C for 10 days in the dark until sporulation. Spores were harvested in sterile mQ water and filtered through glass wool to remove hyphae. Then, they were diluted in Potato Dextrose Broth medium 6 g.L⁻¹ (PDB; BD Biosciences, San Jose,
USA) for inoculation. Five week-old plants were treated as described previously. Then, 3 days after the addition of 25 μM pyoverdine or water, Fe 25-, Fe 25 apo-pyo-, Fe 0- and Fe 0 apo-pyo-treated plants were inoculated by *B. cinerea*. Inoculation was achieved by application of 5 μl droplets of spore suspensions at a concentration of 5.10^4 spores.mL⁻¹ to the upper surface of 5 leaves per plant with 5 plants per condition and per experiment minimum. The inoculated plants were incubated at 100% relative humidity under 16 h day (23°C) and 8 h night (20°C). Symptom development was scored 7 days after inoculation by measuring the lesion area using Optimas version 6.0 (Adept Turnkey Pty. Ltd., West Australia, Australia).

**Statistics**

One-way ANOVA was performed using XLSTAT software (Addinsoft, New York, NY) followed by multiple comparison procedure with the Fisher’s least significant difference (LSD) method (at least P < 0.05). Two-way ANOVA was performed using VassarStats online software (http://vassarstats.net/) followed by multiple comparison procedure with Tukey’s honest significant difference (HSD) method (at least P < 0.05).

**Accession numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL database accession numbers: *IRT1*, AT4G19690; *FRO2*, AT1G015800; *FER1*, AT5G01600; *NAS4*, AT1G56430; *bHLH100*, AT2G41240; *bHLH39*, AT3G56980; *CER4*, AT4G33790; *GRX*, AT3G21460; *LTP3*, AT5G59320; *PLTP*, AT5G59330; *HBII*, AT2G18300.
**FIGURE LEGENDS**

**Figure 1.** Experimental design.

After 4 weeks of hydroponic culture in the nutritive solution containing 25 µM Fe-EDTA, plants were subjected to 24 h pre-treatment in iron-sufficient (nutritive solution with 25 µM Fe-EDTA) or iron-deficient medium (nutritive solution without Fe-EDTA). Then, plants were treated for 7 days with 25 µM apo-pyoverdine in iron-sufficient (Fe 25 apo-pyo) or iron-deficient medium (Fe 0 apo-pyo). As controls, plants were cultivated in iron-sufficient (Fe 25) or iron-deficient medium (Fe 0) without apo-pyo.

**Figure 2.** Detection of apo-pyo in *A. thaliana* tissues.

Plants were cultivated and treated by apo-pyo as indicated in Fig. 1.

A. Apo-pyo was detected in roots by ELISA using rabbit polyclonal anti-pyoverdine antibodies. Signal was expressed in ng of apo-pyo per µg of total root proteins. Each value represents the mean ± SE of three measurements. Experiments were repeated three times with similar results.

B. $^{15}$N-apo-pyo was detected using isotope ratio mass spectrometry in roots and in shoots. Each value represents the mean ± SE of three measurements. Experiments were repeated three times with similar results. DW: dry weight.

**Figure 3.** Phenotypic analysis of *A. thaliana* plantlets exposed to apo-pyo in iron-sufficient or iron-deficient conditions.

Plants were cultivated and treated by apo-pyo as indicated in Fig. 1.

A. Rosette macroscopic phenotypes. Phenotypes were observed 7 days after the addition of apo-pyo. Results are representative of 9 experiments.

B. Root fresh weights.

C. Shoot fresh weights.

D. anthocyanin contents.

Each value represents the mean ± SE of three measurements. Experiments were repeated at least three times with similar results. Significant differences between the treatments were determined by one-way ANOVA/LSD method (P < 0.01 for B, P < 0.0001 for C and D). FW: fresh weight. AU: arbitrary unit.
Figure 4. Venn diagrams illustrating the transcriptome changes in response to apo-pyo in iron-deficient (-Fe) or iron-sufficient (+Fe) medium in roots or in shoots (log 2 ratio $\geq +1.5$ or $\leq -1.5$; p-value $\leq 0.05$).

A. General view of the number of genes commonly regulated between the four comparisons.
B. Focus on the number of genes induced (I) or repressed (R) in the iron-deficient medium.
C. Focus on the number of genes induced (I) or repressed (R) in the iron-sufficient medium.
D. Focus on the number of genes induced (I) or repressed (R) in the roots.
E. Focus on the number of genes induced (I) or repressed (R) in the shoots.

Diagrams were generated using http://bioinfogp.cnb.csic.es/tools/venny/index.html.

Figure 5. Over-represented functional categories of induced and repressed genes relative to the whole genome by annotation for Gene Ontology (GO).

GO Biological process.
GO cellular component.

Induced or repressed genes referred as genes whose expression was highly modulated (log 2 ratio $\geq +3$ or $\leq -3$; p-value $\leq 0.05$) by apo-pyo in iron-deficient medium in roots and in shoots (–Fe root and –Fe shoot).

Figure 6. Iron concentrations measured in the nutritive medium and tissues 6 h and 3 days (3 d) after apo-pyo treatment in iron-sufficient or iron-deficient medium.

A. Iron concentrations measured in the nutritive medium.
B. Root iron contents.
C. Shoot iron contents.

Values are the means of 3 measurements $\pm$ SE from 3 independent experiments. Significant differences between the concentrations of iron for each time point were determined by one-way ANOVA/LSD method, different letters signifying distinct statistical groups (P $< 0.001$ for B and P $< 0.05$ for C).

DW: dry weight.

Figure 7. Growth phenotypes of mutants impaired in genes involved in iron homeostasis in response to apo-pyo in iron-sufficient or in iron-deficient medium after 7 days of treatment.

Plants were treated as described in Fig. 1.

A, C and E. Fresh shoot masses measured in WT and irt1 (A), fro2 (C), nas4 and 35S:NAS4 (E).
B, D and F. Fresh root masses measured in WT and \textit{irt1} (B), \textit{fro2} (D), \textit{nas4} and 35S:\textit{NAS4} (F).

Each value represents the mean ± SE of almost 12 measurements from almost 3 independent biological experiments. Significant differences between the shoot and root masses of WT and of the mutants in response to apo-pyo and/or iron deficiency were determined by two-way ANOVA/Tukey's HSD method (** P < 0.01, *** P < 0.0001). FW: fresh weight.

**Figure 8.** Analysis of hormone contents in the shoots (A, B, C) or in the air (D) 6 h and 3 days (3 d) after apo-pyo treatment in iron-sufficient or iron-deficient medium.

A. SA contents.
B. ABA contents.
C. IAA contents.
D. Ethylene contents.

Values are the means of at least 3 measurements ± SE from at least 3 independent experiments. Significant differences between the concentrations of hormones for each time point were determined by one-way ANOVA/LSD method, different letters signifying distinct statistical groups (P < 0.05 for A and C, P < 0.01 for B).

**Figure 9.** Evaluation of the sensitivity to \textit{Botrytis cinerea} of plants treated by apo-pyo in iron-sufficient or iron-deficient medium.

Each value represents the mean of the area of the necrotic lesion ± SE of 30 measurements. Experiments were repeated four times with similar results. Significant differences between the mean lesion area were determined by a one way ANOVA followed by one-way ANOVA/LSD method, different letters signifying distinct statistical groups (P < 0.05).

**Figure 10.** Growth phenotypes of \textit{HBI1-ox} and \textit{HBI1(L214E)-ox} mutants in response to apo-pyo in iron-sufficient or in iron-deficient medium after 7 days of treatment.

Plants were treated as described in Fig 1.

A. Fresh shoot masses.
B. Fresh root masses.

Each value represents the mean ± SE of almost 12 measurements from almost 3 independent biological experiments. Significant differences between the shoot and root masses of WT and of the mutants in response to apo-pyo and/or iron deficiency were determined by two-way ANOVA/Tukey's HSD method (* P < 0.05). FW: fresh weight.
**Figure 11.** Phenotypic analysis of *A. thaliana* plantlets exposed to apo-pyo or inoculated with C7R12 or PL1 and grown in iron-sufficient or iron-deficient conditions. Plants were cultivated and treated by apo-pyo as indicated in Fig. 1. Inoculation with C7R12 or PL1 was performed as described in the Materials and Methods section.

A. Root fresh weights.
B. Shoot fresh weights.
C. Anthocyanin contents.

Each value represents the mean ± SE of three measurements. Experiments were repeated three times with similar results. Significant differences between the treatments were determined by one-way ANOVA/LSD method (P < 0.05).

FW: fresh weight. AU: arbitrary unit.

**Figure 12.** Sensitivity to *Botrytis cinerea* of plants treated by apo-pyo or inoculated by C7R12 or PL1 bacteria in iron-sufficient or iron-deficient medium.

Each value represents the mean of the area of the necrotic lesion ± SE of 30 measurements. Experiments were repeated three times with similar results. Significant differences between the mean lesion areas were determined by a one way ANOVA followed by one-way ANOVA/LSD method, different letters signifying distinct statistical groups (P < 0.05).
Supplemental data

Supplemental Figure S1. RT-qPCR validation of the results obtained in the microarray analysis.

Plants were treated as described in Fig. 1 and RNAs were extracted from shoots or roots 3 days after apo-pyo treatment.

A. Expression of bHLH39/ORG3 and AT2G38240 in shoots
B. Expression of FRO2, bHLH39/ORG3 and IRT1 in roots

Gene expression was normalized against transcript levels of the housekeeping genes AT5G08290 and AT4G26410 and was expressed in log 2 ratio to enable simple comparison with the CATMA transcriptomic data.

+ Fe: Fe 25 apo-pyo versus Fe 25; - Fe: Fe 25 apo-pyo versus Fe 25.

Supplemental Figure S2. Mapman analysis of genes modulated by apo-pyo in the roots of plants facing iron deficiency.

A. General classification based on cellular responses (log 2 ratio ≥ + 3 or ≤ - 3; p-value ≤ 0.05).
B. Focus on the genes involved in biotic stress responses with log 2 ratio ≥ + 3 or ≤ - 3 (p-value ≤ 0.05).
C. Focus on the genes involved in biotic stress responses with log 2 ratio ≥ + 1.5 or ≤ - 1.5 (p-value ≤ 0.05).

Supplemental Figure S3. Comparison between the profile of expression of the most induced and repressed genes by apo-pyo in the roots in iron-deficiency medium (log 2 ratio ≥ + 3 or ≤ - 3) to the profiles of other transcriptomic data (most different perturbations). The results were generated using the tool “signature” of Genevestigator with the particular condition "hormones".

Supplemental Figure S4. Comparison between the profile of expression of the most induced and repressed genes by apo-pyo in the roots in iron-deficiency medium (log 2 ratio ≥ + 3 or ≤ - 3) to the profiles of other transcriptomic data (most different perturbations). The results were generated using the tool “signature” of Genevestigator with the particular conditions "elicitor" and "biotic".
Supplemental Figure S5. Growth phenotypes of mutants overexpressing or impaired in genes involved in iron homeostasis (bhlh100, bhlh39, ov. fer.) or defense responses or/and growth (cer4, grx, ltp3 and pltp) in response to apo-pyo in iron sufficient or in iron deficient medium.

Plants were treated as described in Fig. 2.

A,B. Fresh shoot and root masses measured in WT, cer4, bhlh100 and bhlh39.
C,D. Fresh shoot and root masses measured in WT and ov. fer.

Each value represents the mean ± SE of almost 12 measurements from almost 3 independent biological experiments. Two-way ANOVA followed by Tukey's HSD revealed no significant difference between the shoot and root masses of WT and of the mutants in response to apo-pyoverdine or iron deficiency (P < 0.05). FW: fresh weight.

Supplemental Figure S6. Comparison between the profile of expression of the genes modulated by apo-pyo in the roots in iron-containing medium (log 2 ratio ≥ + 1.5 or ≤ - 1.5) to the profiles of other transcriptomic data (most similar perturbations).

The results were generated using the tool “signature” of Genevestigator.

Supplemental Figure S7. Rosette macroscopic phenotype of A. thaliana plantlets exposed to apo-pyo or inoculated with the C7R12 or PL1 strains in iron-sufficient or iron-deficient conditions.

Plants were cultivated and treated by apo-pyo as indicated in Fig. 1 or by C7R12 or PL1 bacteria as described in the Materials and Methods section.

A. Phenotypes were observed 7 days after the addition of apo-pyo, C7R12 or PL1 bacteria.
B. Phenotypes were observed 14 days after the addition of apo-pyo, C7R12 or PL1 bacteria.

Results are representative of 3 experiments.

Supplemental Table S1. Genes modulated by apo-pyo (log 2 ratio ≥ + 1.5 or ≤ -1.5) in iron containing medium in the shoots (+ Fe shoots).

Supplemental Table S2. Genes modulated by apo-pyo (log 2 ratio ≥ 1.5 or ≤ -1.5) in iron deficient medium in the shoots (- Fe shoots).
Supplemental Table S3. Genes modulated by apo-pyo (log 2 ratio ≥ 1.5 or ≤ -1.5) in iron containing medium in the roots (+ Fe roots).

Supplemental Table S4. Genes modulated by apo-pyo (log 2 ratio ≥ 1.5 or ≤ -1.5) in iron deficient medium in the roots (- Fe roots).

Supplemental Table S5. Clustering of the genes modulated by apo-pyo in 2 or 3 conditions. Genes in italics are present in almost 2 clusters. Cluster 1: genes modulated in - Fe shoots and - Fe roots; Cluster 2: genes modulated in + Fe roots and - Fe roots; Cluster 3: genes modulated in + Fe shoots and - Fe shoots; Cluster 4: genes modulated in + Fe shoots and - Fe roots; Cluster 5: genes modulated in - Fe shoots and + Fe roots; Cluster 6: genes modulated in + Fe shoots and + Fe roots.

Supplemental Table S6. Genes related to iron homeostasis modulated by apo-pyo (Log 2 ratio ≥ 1.5 or ≤ -1.5) in iron deficient conditions in roots or in shoots. Genes are classified in "response to iron ion", "iron ion homeostasis" or "response to iron ion starvation" in the TAIR database.

Supplemental Table S7. Genes related to defense modulated by apo-pyo (Log 2 ratio ≥ 1.5 or ≤ -1.5) in iron deficient conditions in roots or in shoots. Genes are classified in "response to stress" in the TAIR database.

Supplemental Table S8. Genes related to the trade-off growth/defense mediated by HBI1 modulated by apo-pyo (Log 2 ratio ≥ 1.5 or ≤ -1.5) in iron deficient conditions in roots or in shoots.

Supplemental Table S9. Comparison between the root genes modulated by apo-pyo in iron-containing medium (Log 2 ratio ≤ -1.5 or ≥ + 1.5) and the genes modulated by iron deficiency in roots in the study of Schuler et al. (2011).

Supplemental Table S10. List of the primers used for the characterization of the mutants lines.

Supplemental Table S11. List of the primers used in the RT-qPCR analyses.
ACKNOWLEDGMENTS

We thank Carine Fournier for the preparation of B. cinerea spores, Pascal Tillard and Alain Gojon from the Analytical platform of B&PMP, Cyril Zipfel for the gift of HBI1-ox and HBI1(L214E)-ox lines. We are grateful to Olivier Lamotte, Valérie Nicolas-Francès, Claire Rosnoblet and Hoai-Nam Truong for careful reading of the manuscript.


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C. Shoot fresh weights.

D. Anthocyanin contents.

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Values are the means of 3 measurements ± SE from 3 independent experiments. Significant differences between the concentrations of iron for each time point were determined by one-way ANOVA/LSD method, different letters signifying distinct statistical groups (P < 0.001 for B and P < 0.05 for C).
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Each value represents the mean ± SE of almost 12 measurements from almost 3 independent biological experiments. Significant differences between the shoot and root masses of WT and of the mutants in response to apo-pyo and/or iron deficiency were determined by two-way ANOVA/Tukey’s HSD method (** *P* < 0.01, *** *P* < 0.0001). FW: fresh weight.
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A. Fresh shoot masses.
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A. Root fresh weights.
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